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2. Patent application number *(The Patent Office will fill in this part)* — **9819387.3**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

University College London
Rowland Hill Street
London NW3 2PF

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — 7980520011

4. Title of the invention — POLYPEPTIDE

5. Name of your agent *(if you have one)* — Page White & Farrer

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

54 Doughty Street
London WC1N 2LS

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
Great Britain	9810681.8	18 May 1998

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11. I/We request the grant of a patent on the basis of this application.

Signature Page White & Farrer Date 4 September 1998

PAGE WHITE & FARRER

12. Name and daytime telephone number of person to contact in the United Kingdom — Mr J N Daniels 0171 831 7929

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POLYPEPTIDE

The present invention relates to a polypeptide which is involved in the regulation of phosphate metabolism, to fragments of that polypeptide and to medicaments relating thereto.

Phosphate plays a central role in many of the basic processes essential to the cell and the mineralisation of bone. In particular, skeletal mineralisation is dependent on the regulation of phosphate and calcium in the body and any disturbances in phosphate-calcium homeostasis can have severe repercussions on the integrity of bone. In the kidney, phosphate is lost passively into the glomerular filtrate and is actively reabsorbed via a sodium (Na^+) dependent phosphate cotransporter. The liver, skin and kidney are involved in the conversion of vitamin D3 to its active metabolite, calcitriol, which plays an active role in the maintenance of phosphate balance and bone mineralisation.

Vitamin D deficiency causes rickets in children and osteomalacia in adults. Both conditions are characterised by failure of calcification of osteoid, which is the matrix of bone. There are also several non-dietary conditions which can lead to rickets, including X-linked vitamin D resistant hypophosphataemic rickets (HYP), hereditary hypercalciuria with hypophosphataemic rickets (HHRH), Dent's disease including certain types of renal Fanconi syndrome, renal 1 alpha-hydroxylase deficiency (VDDR I), defects in 1,25-dihydroxy vitamin D3 receptor (end organ resistance, VDDR II), and oncogenic hypophosphataemic osteomalacia (OHO).

Rowe *et al* (1996) have reported candidate 56 and 58 kDa protein(s) responsible for mediating renal defects in OHO [Rowe *et al*, Bone, 18, 159 to 169 (1996)]. A patient with OHO was treated by tumour removal and pre- and post-operative antisera from the patient were used in a Western blotting identification

of tumour conditioned media proteins. Neither the tumour cells nor the antisera were ever made available to the public, however.

In a review in Exp Nephrol, 1997, 5; 335-363, Rowe (1997) discusses the above diseases and the role of the PHEX gene (previously known as the PEX gene). The PHEX gene product has been identified as a zinc metalloproteinase and Rowe has postulated the existence of a new hormone, phosphatonin, the role of which is to regulate phosphate metabolism. As set out in attached Figure 6, Rowe has postulated that in healthy individuals phosphatonin is cleaved by the PHEX metalloproteinase and a cleaved fragment of phosphatonin binds a renal receptor. In disease states such as familial rickets, defective PHEX results in uncleaved phosphatonin which would result in down regulation of the sodium dependent phosphate cotransporter and up regulation of renal mitocondrial 24-hydroxylase. It has been speculated that a possible candidate for the phosphatonin hormone is stanniocalcin, which is known from fish. Rowe (1997) indicates that the presence of a stanniocalcin-like hormone in human kidney has been confirmed by the use of fish stanniocalcin antibodies [Wagner *et al*, PNAS (1995) 92, 1871 to 1875]. No purification of phosphatonin or stanniocalcin was reported by Rowe (1997).

In one aspect, the present invention provides an isolated polypeptide having phosphatonin activity. Prior to the present invention no source material for phosphatonin was made available to the public. Moreover, purification, identification and characterisation of phosphatonin has not been possible.

The polypeptide typically has an approximate molecular weight of 53 to 60 kDa, more preferably 58-60 kDa, as measured on SDS-PAGE, particularly on a 12.5% gel at pH8.6 in TRIS-Glycine SDS buffer. An approximate molecular weight of 200 kDa may be measured on bis- tris-SDS-PAGE at pH7 using a 4-12% gradient gel

with MOPS running buffer. It is possible on such a gel also to see lower molecular weight bands of 53 to 60 kDa. The polypeptide is generally glycosylated, and preferably comprises phosphatonin in substantially pure form.

Surprisingly, it has been found that the phosphatonin is obtainable, following purification from Saos-2 cells, which are available from the European Collection of Cell Culture under Deposit No. ECACC 89050205. Accordingly, in a further aspect of the invention, there is provided use of Saos-2 cells or HTB-96 cells for the production of phosphatonin. Other transformed or immortalised cell lines may be capable of overexpression of phosphatonin, such as transformed osteoblast or bone cell lines.

As set out in further detail below, a polynucleotide has been isolated which encodes polypeptides according to the present invention. The amino acid and nucleotide sequences of phosphatonin are set out in Figure 8. Accordingly, the polypeptide of the present invention comprises the amino acid sequence of Figure 8, optionally including mutations or deletions which do not substantially affect the activity thereof. Such mutations include substitution of one or more amino acids, particularly by homologues thereof, as well as additions of one or more amino acids, especially at the N or C termini. Deletions include deletions from the N or C termini. Substitutions by both naturally-occurring and synthetic amino acids are possible. Also included are polypeptides modified by chemical modification or enzymatic modification.

Phosphatonin activity may be measured by routine assay, particularly as the ability to down-regulate renal sodium dependent phosphate co-transport and/or up-regulate renal 25-hydroxy vitamin D3-24-hydroxylase and/or down-regulate renal 25-hydroxy-D-1 α-hydroxylase. In each case, regulation of the relevant enzyme activity may be effected directly or indirectly by the phosphatonin. These activities may be assayed using a

suitable renal cell line such as CL8 or OK (deposited at the European Collection of Cell Cultures under ECACC91021202). A suitable assay methodology is found in Rowe *et al* (1996). Phosphatonin activity may further be measured by the ability to promote osteoblast-mediated mineralisation in tissue culture: Santibanez, J.F., Silva, S., and Martinez, J. (1996). Soluble factors produced by PC-3 prostate cells decrease collagen content and mineralisation rate in fetal rate osteoblasts in culture. Br.J.Cancer *74*, 418-422. Stringa, E., Filanti, C., Giunciuglio, D., Albini, A., and Manduca, P. (1995). Osteoblastic cells from rat long bone. I. Characterization of their differentiation in culture. Bone *16*, 663-670. Aronow, M.A., Gerstenfeld, L.C., Owen, T.A., Tassinari, M.S., Stein, G.S., and Lian, J.B. (1990). Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. J.Cell Physiol. *143*, 213-221.

In a further aspect, the present invention provides a polypeptide comprising a bioactive fragment of the polypeptide described above. It is thought that phosphatonin may function as a polyhormone which may be cleaved *in vivo* to form one or more fragments at least some of which possess biological activity such as hormonal activity. *In vivo* it is thought that phosphatonin may be cleaved proteolytically, for example by the PHEX gene product to produce at least one functional fragment. In a preferred embodiment, the polypeptide comprising the bioactive fragment is capable of regulating phosphate metabolism, for example by possessing phosphatonin activity as discussed above, or by possessing the opposite of phosphatonin activity as discussed in further detail below. The bioactive fragment may be an N-terminal, C-terminal or internal fragment. The polypeptide comprising the bioactive fragment may further comprise additionally amino acid sequence provided that the activity of the bioactive fragment is not substantially affected.

Advantageously, the bioactive fragment has a cell attachment motif which preferably comprises RGD. As discussed in further detail below, this motif may be involved in receptor and/or bone mineral matrix interaction. Advantageously, the bioactive fragment has a glycosaminoglycan attachment motif, which preferably comprises SGDG. Attachment of glycosaminoglycan is thought to permit the fragment to resemble a proteoglycan. Proteoglycans are known to be involved in bone bioactivity, particularly in cell signalling. These motifs are discussed in greater detail below.

In one embodiment of the present invention, the polypeptide comprising the bioactive fragment possesses phosphatonin activity. Such activity is expected in phosphatonin uncleaved by PHEX metalloproteinase and some bioactive fragments carrying a PHEX metalloproteinase cleavage site such as the site ADAVDVS where cleavage is proposed to occur between residues VD (residues 235 and 236. The bioactive fragment may comprise at least the first 236 residues of the amino acid sequence of Figure 8 so that this PHEX metalloproteinase cleavage site is part of the fragment. Such polypeptides and fragments thereof having phosphatonin activity will be useful in treating hyperphosphataemic conditions.

In a further embodiment of the present invention, the polypeptide comprising the bioactive fragment has the reverse of phosphatonin activity and may be suitable for treating hypophosphataemic conditions. In this embodiment, the polypeptide is directly or indirectly capable of up-regulating renal sodium dependent phosphate cotransport and/or down-regulating 25-hydroxy vitamin D3-24-hydroxylase and/or up-regulating renal 25-hydroxy-D-1 -hydroxylase. These activities are also readily measurable using the methodology of Rowe *et al* (1996) by assay using a suitable renal cell line such as CL8 or OK (deposited at the European Collection of Cell Cultures under ECACC 91021202). Preferably, the fragment is obtainable by

proteolytic cleavage of phosphatonin by a PHEX metallopeptidase. A PHEX gene has been cloned and found to encode a zinc metallopeptidase as discussed in Rowe (1997). Structurally, bioactive fragments having these activities are thought to lack at least a part of the C terminal portion of the amino acid sequence of Figure 8, preferably lacking the C terminal portion up to at least the putative PHEX metalloproteinase cleavage site at residues 235/236. This polypeptide therefore preferably comprises no more than approximately the first 235 residues of the amino acid sequence of Figure 8.

In a further aspect, the present invention provides the polypeptide or a fragment thereof, for use as a medicament. In particular, bioactive fragments as described above may be useful as a medicament in the treatment of a disorder of phosphate metabolism such as X-linked rickets and osteomalacia as well as other diseases of bone mineral metabolism. There is further provided phosphatonin and PHEX metallopeptidase as a combined preparation for simultaneous, separate or sequential use as a medicament. In this way, the PHEX metallopeptidase may be used to cleave phosphatonin so as to produce active phosphatonin fragments which may be used for the treatment of disorders of phosphate metabolism as discussed herein. Whilst all of these diseases are particularly important in humans, other mammals may also be treated in accordance with the invention.

The invention further provides a pharmaceutical composition comprising a physiologically acceptable exipient, carrier or diluent and, as active ingredient, a molecule according to the invention. The molecule, and pharmaceutical compositions thereof of this invention may be administered by any route but preferably are for parenteral administration such as subcutaneous, intramuscular or intravenous administration.

The present invention has therefore provided for the first time phosphatonin in a substantially isolated or purified form which

is suitably free of contaminants. Native phosphatonin and native fragments of phosphatonin, which are free of contaminants such as SDS and/or other interfering proteins are capable of regulating phosphate metabolism and of providing active ingredients in pharmaceutical compositions for the treatment of diseases associated with disorders of phosphate metabolism.

The present invention further provides a polynucleotide encoding a polypeptide as described herein. Such polynucleotide may be a DNA such as a cDNA, an RNA such as mRNA or any other form of nucleic acid including synthetic or modified derivatives and may encode the polypeptide in a continuous sequence or in a number of sequences interrupted by intervening sequences. In which ever form it is present, the polynucleotide is an isolated polynucleotide in that it is removed from its naturally-occurring state. This aspect of the invention is based on the cloning of the gene for human phosphatonin. In a preferred embodiment, the polynucleotide comprises the nucleotide sequence of Figure 8, optionally including one or more mutations or deletions which do not substantially affect the activity of the polypeptide encoded thereby. Such mutations include those arising from the degeneracy of the genetic code, as well as those giving rise to any of the amino acid mutations or deletions discussed above. Accordingly, by the employment of techniques routine to those skilled in molecular biology, it is possible to use the nucleotide sequence of Figure 8 to generate suitable polynucleotide sequences which encode polypeptides useful in the present invention.

In a further aspect, the present invention provides a recombinant polynucleotide comprising a vector incorporating the polynucleotide of the present invention. Many suitable vectors are known to those skilled in molecular biology, the choice of which would depend on the function desired. As discussed in further detail below, a cloning vector was used to isolate individual sequences of DNA. Relevant sequences can be

transferred into expression vectors where expression of a particular polypeptide is required. Typical cloning vectors include pBscpt sk, pGEM, pUC9, pBR322 and pGBT9. Typical expression vectors include pTRE, pCAL-n-EK, pESP-1, pOP13CAT.

In a further aspect, the present invention provides a cell capable of expressing a polypeptide as discussed herein. The cell comprises a recombinant host cell incorporating the polynucleotide. Preferably, the host cell incorporates the polynucleotide as the recombinant polynucleotide. Any suitable host cell may be chosen, again depending on the intended purpose. Suitable host cells include XLI-BLUE, B21(DE3)pLysS, HB101, SOLR and SP-Q01 (Saccharomyces pombe).

Using an appropriate combination of host cell, vector and polynucleotide, an expression system can be provided so as to obtain a polypeptide useful in the present invention. This may comprise a fusion polypeptide encoded by the recombinant polynucleotide, a part of which is encoded by the vector. Typically, the vector will have a promoter region, which is usually inducible, leading to a 5' coding region associated with the promoter. By appropriate manipulation, the polynucleotide encoding the polypeptide can be attached to the 5' coding region in frame. In this way, expression of the nucleotide sequence downstream of the promoter region gives rise to the fusion polypeptide which includes the polypeptide of the present invention.

The present invention will now be described in further detail, by way of example only, with reference to the accompanying drawings, in which:

FIGURE 1 shows a photomicrograph of an immortalised tumour cell line capable of expressing phosphatonin;

FIGURE 2(a) and (b) show respectively chromatograms with low affinity and high affinity protein-containing peaks from a concanavilin A column;
FIGURE 3 shows a cation exchange chromatogram of fractions from the concanavilin A column;
FIGURE 4 shows results of Western blotting of material from the concanavilin A column;
FIGURE 5 shows results of Western blotting and glycoprotein detection of tumour condition medium and purified fractions using enhanced chemiluminescence;
FIGURE 6 shows the results of Western blotting on an SDS-PAGE polyacrylamide gradient gel;
FIGURE 7 shows a simplified scheme for the roles of phosphatonin and PHEX in phosphate metabolism;
FIGURE 8 shows the amino acid and nucleotide sequences of phosphatonin;
FIGURE 9 shows a computer prediction of hydrophilicity and hydrophobicity of phosphatonin;
FIGURE 10 shows a computer prediction of antigenicity of phosphatonin;
FIGURE 11 shows a computer prediction of flexibility of phosphatonin;
FIGURE 12 shows a computer prediction of surface probability of the secondary structure of phosphatonin; and
FIGURE 13 shows a computer prediction of the secondary structure of phosphatonin.

Example 1

Purification of Phosphatonin from Tumour

A mesenchymal tumour with phosphaturic expression was removed from a patient and the following samples taken:

A: Sample of pure tumour tissue, size of two large peas, was placed into a 2 ml vial containing DMEM Eagles/ 10%FCS/Glutamine/antibiotic antimycotic Gibco-BRL.

B: Sample of sub-dura tumour approximately the same size possibly smaller. Placed in same media as A.

C: Sample of abnormal dura: tough white material: Placed in same media as A.

D: Sample of tumour fluid.

Processing of Samples:

DAY 1

The samples were each cut into small 0.5 cm cubes using a sterile scalpel. Half of each sample was placed into a cryotube and frozen down in N2(l) immediately. The fluid surrounding the tissue (DMEM/10% FCS etc.), was also collected and frozen down. The other half of each sample was added to DMEM Eagles/10% FCS/Glutamine/Antimycotic antibiotic supplemented with collagenase A1 0.2mg/ml (~15ml), and left at 37 C O/N.

DAY 2

1. After overnight incubation in serum supplemented DMEM, the cells appeared to be predominantly RBC's and very few adherent cells were observed. The cells were spun down at room temp and the supernatants collected and immediately frozen down (~15ml).

2. The pellets were then resuspended in 10 ml of DMEM Eagles supplemented with antibiotic/antimycotic (medium flasks), and then incubated for a further 8h 10 min.

3. The serum-free supernatants were collected as described in 1 (~10 ml), and the cells were resuspended in DMEM EAGs with 10% FCS etc., (~15 ml), and incubation continued. The supernatants were stored at -80°C.

DAY 6

1. After incubation from Day 2, cells were spun down as described for 1 of Day 2. 10% FCS samples were collected and frozen.

2. Pellets were resuspended in serum free DMEM (10 ml), as for Day 2. and this time left for four hours.

3. Same as for 3 of Day 2.

DAY 7:

1. The subdura and tumour culture in particular, had developed innumerable foci containing clumps of cells which appeared attached to the plastic of the tissue culture plates. Underneath these polyp like protuberances was a monolayer of fibroblast like cells which spread out radially from underneath the tumour like structures. This layer of cells appeared to act as a matrix to anchor the polyp like tumours. None of this was seen in the dura sample, which appeared to lack cells at this stage, and contained fibrous like matted structures.

2. Cultures were spun down, and the supernatants collected (10% FCS). The pellets were then placed to one side.

3. The plates were then incubated with 10 ml of trypsin EDTA soln Gibco/BRL 1/10 dilution in PBS for ~15 min. Plates were then tapped vigorously and 5 ml of FCS added.

4. The resuspended cells were then added to the pellets obtained in 2, resuspended and spun down. The supernatant was discarded.

5. Cells were then plated out in 18 ml of 10 % FCS DMEM Eagles medium with glutamine and antibiotic antimycotic supplements (large flasks were used).

6. Finally cells were incubated at 37 C in a CO2 atmosphere.

DAY 9:

1. Tumour cells and to some extent the subdura cells appeared as innumerable clumps of cells, and appeared to have the same morphology as the cells prior to trypsin treatment. Some of the clumps were quite large, and visible to the naked eye.

2. The serum supplemented media was collected and stored down. Large flasks were used and 18 ml of media per flask added (DMEM 10% FCS antimycoctic/antibiotic/glutamine).

DAY 13:

1. Cells were frozen down (~15 ml), and stored in falcons as 10%FCS DMEM conditioned media.

2. Cells resuspended in serum free DMEM Eags (~11 ml) 11.10 am, and left for 6 h at 37°C (CO2 incubator).

3. Cells were then spun down and the supernatants collected (serum free control media). 10% FCS DMEM EAG was then added to the remaining cells.

DAY 16

The above process was repeated and Tumour Conditioned Medium (TCM) collected over several weeks.

Alternatively, TCM may be collected from Saos-2 cells (ECACC 89050205) or U-2 OS cells (ATCC HTB-96).

Purification of phosphatonin:

Concanavilin A sepharose affinity chromatography:

1. 3ml of TCM was adjusted with 1M sodium phosphate pH 7.2 and 5M NaCl to give a final concentration *of* 0.06M Sodium phosphate pH 7.2 and 0.5M NaCl plus 0.01% sodium azide.

2. Con A Sepharose (Pharmacia Code No: 17-0440-01), arrived in 20% Ethanol, and this was first washed with several column volumes of water, and then equilibrated in the running buffer. A small C10/10 column (Pharmacia code No: C10/10 id 10 mm), was packed with Con A to a height of 5.5 cm (approx. volume 4.3 to 5.0 ml). Equilibration was carried out at max flow rate of 0.5 ml/min.
3. The sample (adjusted to pH 7.2 sodium phosphate/0.5M NaCl/0.01% sodium azide), was then added to the column by gravity feed, and reloaded three times. The colour of the sample enabled visualisation of the passage through the column. Unbound material was then collected and stored for future reference.
4. Waters LC system was then connected and the sample was washed with several column volumes of loading buffer.
5. After loading and washing, elution was carried out using sodium phosphate buffer 60 mM pH 7.2/ 0.5M Nacl/0.5M a-methyl-D-glucopyranoside/0.01% azide buffer. See Figure 2a. A single peak was detected and this was collected.
6. The column was then run to base line approximately 40 ml max, and then left overnight.
7. After O/n incubation in methyl glycoside buffer, a second peak was eluted (see Figure 2b), which peaked at ~5 ml.
8. The second peak was collected and dialysed against 0.05M acetic acid, and then lyophilised. Both Conacanavilin peaks A1 (low affinity), and concanavilin A2 (high affinity), are potent at inhibiting Na+ dependent phosphate co-transport and vitamin D metabolism in a human renal cell line (CL8). The high affinity fraction The human renal cell line (CL8), and the conditions used for assay are described in Rowe et al 1996. A further suitable known renal cell line for this assay is the OK cell line deposited as ECACC 91021202.

Cation exchange Chromatography using HiTrap SP cation exchange 1 ml column

(Code No 17-1151-01; Pharmacia):

1. The lyophilised protein was then re-dissolved in 0.05M ammonium acetate pH 5 and the applied to an equilibrated 1 ml HiTrap SP sepharose cation exchange column.
2. The column was equilibrated prior to sample addition by washing with water, and then 5 volumes of start buffer (0.02 M ammonium acetate pH 5).
3. Sample was eluted using the following protocol;

Num	Time min	Flow rate ml/min	%NH_4 acetate pH 5	% NH_4 acetate/ 0.5M NaCl pH 5
1		0.5	100	0
2	15	0.5	25	75
3	20	0.5	0	100
4	25	0.5	0	100
5	35	0.5	100	0
6	50	0.5	100	0

A single sharp peak was obtained, and the sample was then dialysed against 0.05M acetic acid and lyophilised. See Figure 3.

After resuspending in 10 mM phosphate buffer pH 7.2 20 ul, aliquots were resuspended in SDS-PAGE sample buffer (to a final concentration = 125mM TRIS-HCL pH6; 2.5% glycerol; 0.5% w/v SDS: 5% β-mercaptoethanol; 0.01% bromophenol blue), boiled (5 mins), cooled and then run on an SDS PAGE gel 12.5% (see chromatogram), and a double band of 55 KD was resolved (see Rowe *et al* 1996). Both the Concanavilin A and cation bands also have an aggregated form. All fractions including the tumour conditioned media were potent at inhibiting Na+dependent phosphate co-transport in a human renal cell line (1/1000 diln), and also altered vitamin D metabolism. For a full description of the methods used to measure phosphate transport and vitamin D metabolism see Rowe *et al* 1996. All purification modalities were carried out on a waters HPLC/FPLC system programmed by computer-millennium

software. The most active fraction was the concanavilin A1 peak shown in figure 4, which shows Western (chemiluminescence) of purified concanavilin A1 fraction from OHO tumour. Anti pre-operation antisera was used to screen the immobilised purified fraction. The fraction is also potent at inhibiting NaPI, and affects vitamin D metabolism in a human renal cell line (CL8).

Screening of tumour conditioned-medium (TCM), and purified fractions with pre/post- operation antisera: plus glycoprotein screen.

Pre-operation and post-operation antisera from a patient has been described previously in Rowe *et al* 1996. Only pre-operation antisera detected the purified fractions and hormone in TCM as shown in figure 5 in which Western and glycoprotein detection of TCM and purified fractions was achieved using enhanced chemiluminescence. Protein markers were biotinylated, and tagged with strepavidin peroxidase conjugate . The arrows show the aggregate and active glycoprotein. Post-operation antisera and rabbit pre-immune sera did not detect any of the fractions. Also, only those tumours secreting phosphaturic factor were positive. Media and skin controls were negative. A distinct feature of the Con A1, Con A2 and CA1 samples was their potent ability to inhibit NaPI, and alter vitamin D metabolism in a human renal cell line (CL8). All the purified fractions have a tendency to aggregate into a lower mobility form on SDS-PAGE, as seen in figure 5. Also, the purified fractions and TCM active fractions are heavily glycosylated. The extent of glycosylation was confirmed by periodate oxidation of immobilised proteins on PVDF membranes followed by biotinylation of carbohydrate moieties. These were then screened with streptavidin conjugated to horse radish peroxidase and enhanced chemiluminescence (figure 5). The active form (inhibits NAPI etc.), is associated with the 58 to 60 kDa fraction shown in figure 5. An additional and powerful way of purifying the protein to homogeneity is the use of a neutral pH

7 SDS-PAGE system using a 4-12% Bis-Tris Gel with MOPS running buffer. Pre-caste gels can be purchased from Novex.

SDS-PAGE at neutral pH using 4-12% polyacrylamide gradient and Bis-Tris gel with MOPS running buffer (Nu-PAGE system from NOVEX): Reduced mobility of hormone.

On this system a fraction of the glycosylated hormone has a reduced mobility, and runs at ~200 kDa. The lower molecular weight form is also visible at 58/60 kDa. Appearance of the ~200kDa protein may be due to the isoelectric point of the protein (different charge at neutral pH), and the interaction of carbohydrate moiety with the gel matrix. Also, increased efficiency of electro-blotting of high molecular weight components occurs due to the low % acrylamide (4-12% gradient), at the top of the gradient gel. Running fractions through this system increases the purity and homogeneity of the molecule. Figure 6 shows a Western blot using this system and includes the following samples (pre-operation antiserum was used to screen the blots using enhanced chemiluminescence detection): 1: protein markers; 2. intracranial tumour cell line OHO; 3 cells from sub-dura adjacent to tumour; 4. Cells from dura adjacent to sub-dura; 5. HTB6 cell line; 6. Saos-2 cell line; 7. defined medium control; 8. Skin fibroblast control; 9. Linear seabaceous naevus polyp tumour. The specific phosphaturic band at ~200kDa on SDS-PAGE Neutral gels is highlighted in the Figure. The above is representative; many control experiments have been done using a number of tumours (phosphaturic/non-phosphaturic).

Example 2

Cloning and sequencing of phosphatonin and tumour-associated cytotoxic factor, clusterin:

1.*Library construction:*

A tumour derived from a patient described in our earlier publication (Rowe et al., 1996), was sectioned and mRNA extracted using standard techniques. The mRNA was copied using reverse transcriptase to generate a cDNA population that was then subsequently subcloned into a bacteriophage vector λ-ZAP II uni (vector purchased from Stratagene Ltd, Unit 140, Cambridge Science Park, Milton Road, Cambridge, CB4 4GF United Kingdom). The cloning was uni-directional and the 5′ end of the gene was adjacent to the T3 promotor and abutted an EcoRI site. The 3′ end of the cDNA's abutted an XHO-1 site upstream of a bacterial T7 promotor.

2.Screening with pre-operative antisera:

The cDNA bacteriophage library was plated out on NZY agar plates and the β-galactosidase operon induced using IPTG. Expressed fusion proteins were then transferred to hybond-C membranes (Amersham) and the membranes were then screened with pre-operation antisera from the patient. The antisera used has been described (Rowe et al., 1996). Prior to use the antisera was extensively pre-absorbed with E.coli lysate, and whole blood to reduce signal to noise. After screening ~600,000 clones, nine positives were selected and purified by secondary and tertiary screening. The bacteriphage clones were rescued as phagemids using ExAssist helper phage and cloned into E.coli SOLR cells. ExAssist helper phage and SOLR cells were purchased from Stratagene Ltd, Unit 140, Cambridge Science Park, Milton Road, Cambridge, CB4 4GF, United Kingdom.

2.Sequencing clone:

Phagemids were prepared and the DNA sequenced. All nine clones were sequenced. Six of the clones were overlapping and in frame with the bacterial β-galactosidase promotor to give contiguous/overlapping epitopes and expressed proteins with identical overlapping DNA sequences. The longest sequenced clone encompassed the cDNA sequences of the five others and is shown in Figure 8. This sequence (amino acid/cDNA) is a complete sequence for phosphatonin. There are 493 amino acid residues cloned and 1655 bp of DNA sequence. Secondary structure prediction indicates a highly hydrophilic protein with glycosylation at the COOH end, and the presence of a cell attachment tripeptide at the amino end (RGD), see Figure 8. The protein is also highly antigenic with a number of major helical domains (Figure 10). Extensive screening of all available databases using BLAST has not revealed any statistically relevant homology to known genes or protein sequences.

The remaining 3 cDNA clones are also overlapping and are in frame with the bacterial β-galactosidase promotor, but are 100% homologous to clusterin (Database accession 116533, Swiss-Prot accession P10909, and Prosite accession PS00492). Clusterin has been described as a suicide protein associated with programmed cell death. This fits into our previous observations (Rowe et al., 1996), where we described the cytoxicity of tumour conditioned media (TCM), in the absence of serum (human renal cell line CL8). Clusterin is also glycosylated and is expressed in a variety of tissues. A major property of clusterin is that it binds to cells, membranes and hydrophobic proteins. The binding of clusterin to hydrophobic proteins explains our observations that foetal calf serum, is able to inhibit in a

dose dependent manner the cytoxicity of TCM (we now know to be clusterin-mediated).

3. Purification of recombinant human phosphatonin:

The cDNA clones isolated are represented as rescued phagemids in Bscpt SKII- vector (Stratagene vector), and contained within SOLR E.coli host cells. Low level fusion protein expression via induction of the β-galactosidase promotor by IPTG has been achieved. Both clusterin and phosphatonin clones fusion-products react with pre-operation antisera on western blots. Increased expression and bioactivity of the fusion proteins can be achieved by sub-cloning into the pCAL-n-EK vector (Stratgene vector) (see below). The construct containing human phosphatonin is contained in E.coli (BL21(DE3) pLysS) cells (purchased from Stratagene). IPTG induction of fusion protein is much higher, and essentially pure protein can be obtained by calmodulin affinity-chromatography of cell lysates. Recombinant phosphatonin with fusion-tag binds to the calmodulin resin in the presence of Ca^{2+}. Phosphatonin fusion protein is then released after washing with EGTA. The small microbial fusion-tag is removed by treatment with enterokinase, leaving pure human phosphatonin. This molecule is predicted to be bioactive (inhibits renal Na-dependent phosphate co-transport, increases 24 hydroxylase gene expression, decreases 1 alpha hydroxylase gene expression, inhibits bone mineralisation and osteoblast function, affects directly/indirectly phosphorylation and gene expression of target bone mineral proteins such as osteopontin and osteocalcin, and also collagen proteoglycan cell attachment interactions).

3a. *Subcloning Phosphatonin into pCAL-n-EK vector*

The Phosphatonin cDNA sequence in frame with the primary amino acid sequence may be cloned into the vector using PCR and ligation independent cloning. Two PCR primers complementary to the 5′ end of the gene, and 3′ end of the gene are prepared. Both contain linker sequences overlapping prepared pCAL-n-EK plasmid, and suitable for cloning into the vector as follows:

Forward 5′ ***GACGACGACAAG***.GTGAATAAAGAATATAGTATCAGTAA 3′
Linker

Reverse 5′ ***GGAACAAGACCCGT***.CTAGTCACCATCGCTCTCACT 3′
Linker

PCR amplification of phosphatonin will include DNA sequence coding for the first valine residue to the stop codon of phosphatonin (see Figure 8), plus linker sequence. A 5′ overhang of the linker sequence will then be generated by treating the PCR fragment with Pfu polymerase and dATP. The fragment will then be directly cloned into pCAL-n-EK vector and transformed into E.coli BL21(DE3)pLysS cells. Induction of fusion protein will then be carried by growing the cells and adding IPTG.

3b. *Purifying phosphatonin by calmodulin affinity resin*

The method described by Stratagene (cat~214405), may be used. Sequence upstream from the phosphatonin specific residues will contain a calmodulin binding sequence. Calmodulin resin is added to the crude cell lysate in the presence of calcium, and the protein allowed to bind. The slurry is then washed with calcium containing buffer, and the phosphatonin fusion protein eluted by addition of EGTA

2 mM in a Tris buffer (50 mM Tris-HCl pH 8). Removal of the calmodulin binding protein tag is then accomplished by digestion with site-specific protease EK, leaving pure recombinant human phosphatonin.

4. Summary

We have cloned a phosphaturic factor and have a complete amino acid and cDNA sequence (see Figure 8). Recombinant phosphatonin has been produced in E.Coli. Also, we have shown that clusterin is secreted by the tumour and thus may play a role in the pathophysiology of the tumour associated disease.

Structure of phosphatonin

1. Primary structure and motifs:

The primary structure of the protein and the nucleic acid sequence are shown in Figure 8. The protein has glycosylation motifs at residues 382 to 385 (NNST), and residues 383-386 (NSTR). There is also a glycosaminoglycan attachment site at residues 161-164 (SGDG). The approximate molecular weight without glycosylation is 54 kDa, and is in close agreement with the purified glycosylated form of (58-60 kDa). There are a number of phosphorylation site motifs (see Table 1), and these are predicted to play a role in the biological activity of the hormone or fragments thereof.

A key feature of the protein is a cell attachment sequence at residues 152-154 (RGD). The Arg-Gly-Asp sequence plays a role in receptor interactions, and in fibronectin is essential for cell surface receptor binding to a specific integrin. More notable is the presence of this motif in some forms of collagens (bone matrix protein), fibrinogen, vitronectin, von Willebrand

factor (VWF), snake disintegrins, and slime mould discoidins. It is highly probable that this part of the phosphatonin is involved in receptor and/or bone mineral matrix interactions. Also these interactions mediate the following:

1. osteoid mineralisation (osteoblasts).
2. Na-dependent phosphate co-transporter gene expression (kidney).
3. 24 hydroxylase and/or 1 alpha hydroxylase gene expression (kidney).
4. bone mineral matrix interactions and mineral deposition via nucleation.

The presence of a glycosaminoglycan attachment sequence at residues 161-164 (SGDG), has important implications concerning bone mineral attachment and interactions. The role of proteoglycans in bone is well documented particularly in cell signalling. It is highly probable that this part of the molecule is also essential for the above bioactivities (points 1 to 4), and in particular osteoblast mediated mineralisation of osteoid.

2. Secondary structure:

GCG peptide structure prediction profiles of hydrophobicity/hydrophilicity, antigenicity, flexibility and cell surface probability are shown in Figures 9 to 12. These Figures show GCG-peptidestructure prediction analysis of the primary amino acid sequence. Hydrophobicity and hydrophilicity indices are represented as triangles and ovals respectively. Glycosylation motifs are represented as circles on stalks at residues 382-386. Glycosylation symbols can be seen more clearly in Figure 12. Protein turn is indicated by the shape of the line representing primary amino acid sequence. Regions of

α-helix, coil and sheet structure are indicated by localised undulations of the line (refer to Figure 13 for more detail). Computer predictions were made using GCG-software derived from HGMP resource centre Cambridge (Rice, 1995) Programme Manual for the EGCG package. (Cambridge, CB10 1RQ, England: Hinxton Hall). A striking feature is the lack of cysteine residues and the high degree of hydrophilicity, with four minor sites with low hydrophobic indices (residues 48-53, 59-70, 82-89, and 234-241). The protein does not have a transmembranous profile as deduced from a secondary structure prediction using antheroplot software. The protein is also highly antigenic and flexible (Figures 10 and 11). The overall secondary structure profile is indicative of an extracellular secreted protein, and is in agreement with the proposed function of the molecule. Figure 13 shows the helical, sheet structure, turn and coil regions of the phosphatonin. This is based on a prediction using Garnier analysis of the antheplot v2.5e package. The four lines in each section (top to bottom), represent helix, coil, sheet, and turn probability indices of primary amino acid sequence. The graph at the bottom presents the same data in block form. Notable is the high helical content, particularly at the NH2 terminus and also towards the C-terminus, which may have a functional context.

Cleavage and postranslational modification of phosphatonin: polyhormone

Phosphatonin and proteolytically cleaved derivative-fragments, function as indispensable regulators of the gene expression of Na-dependent phosphate co-transporters (proximal convoluted tubules of the kidney), renal 24 hydroxylase, and 1 α hydroxylase. Also, the mineralisation of osteoid and specific

bone mineral related functions of osteoblasts are also dependent on this polyhormone. Bone mineral matrix proteins such as osteopontin, and osteocalcin, are also proposed to be regulated directly or indirectly by phosphatonin or derivative bioactive peptides. The recent cloning of a gene (PHEX), defective in X-linked hypophosphataemic rickets indicates that a novel phosphaturic factor is processed by the PHEX gene product (Rowe, 1997). The catalytic site of PHEX (a zinc metalloendopeptidase), was deduced after a detailed mutation-analysis and computer secondary structure prediction profile of 99 affected families (Rowe *et al*, 1997b, Human Mol.Genet, 1997, 6, 539-549).

The overlapping pathophysiology of tumour osteomalacia and X-linked rickets, strongly indicates that tumour-derived phosphatonin described herein is post-translationally modified by PHEX. The site of phosphatonin cleavage by PHEX can be predicted using the data described for the PHEX catalytic site (Rowe, 1997). A strong candidate region for cleavage by PHEX occurs at the hydrophobic site 232-238 (....ADAVDVS...), and cleavage is proposed to occur between residues VD to generate ...ADAV DVSLVEG... Additional cleavage is also likely to occur and the fragments generated would have disparate functions relating to phosphate, vitamin D and bone mineral metabolism.

Example 3

Medical Uses of Phospahatonin and Phosphatonin Fragments

A number of disorders are amenable to treatment using polypeptides according to the present invention.

X-linked rickets (hypophosphataemia) (HYP):

X-linked hypophosphataemic rickets is one of the commonest inherited diseases of bone mineral metabolism (Rowe, 1997).

Phosphatonin bioactive fragments such as those cleaved by PHEX and the uncleaved hormone will play a major role in the treatment of the disease. The protein cloned and described herein, is predicted to interact with its cognate receptor in the kidney and cause an inhibition in the expression of a renal Na-dependent phosphate co-transporter (NaPI), and either directly or indirectly up-regulation of a renal 24 hydroxylase. It is also predicted to down regulate expression of renal 1 α hydroxylase (directly/indirectly). After cleavage with PHEX or other post-translational modifiers, the peptide fragments derivative of the hormone are predicted to have the opposite bio-function (up-regulation of NaPI, down-regulation of 24 hydroxylase, up regulation of 1 alpha hydroxylase). The fragment containing the RGD cell attachment residue (152-154), is predicted to play a role in the receptor interactions, although other peptide derivatives may also mediate receptor ligand interactions for disparate bioactivities. Also, phosphatonin derivatives will play an important function in the normalisation of the hypomineralised bone lesions. This is predicted to occur by mediating changes in the osteoblast mediated mineralisation of osteoid, and by correcting the aberrant expression/phosphorylation of bone mineral matrix proteins (osteopontin/osteocalcin). The RGD cell attachment sequence and also the glycosaminoglycan attachment motif could be required for the functional nucleation and crystallisation of hydroxyapatite and bone mineral.

Growth impairment is a major feature of HYP, and current treatments are unsuitable. Treatment by administration of phosphatonin-derived fragments as opposed to inorganic phosphate and vitamin D supplementation, may correct this.

Accordingly, among the useful effects of peptide fragments of phosphatonin are:

1. Correction of hypophosphataemia (NaPi renal)
2. Normalisation of 24-hydroxylase 1 alpha hydroxylase activity (renal).
3. Mineralisation of bone and bone repair (correction/prevention of rickets).
4. Complete loss of bone pain symptoms.
5. Correction of stunted growth.

Oncogenic hypophosphataemic osteomalacia (OHO):

The clinical profile of OHO is similar to HYP. There is a renal phosphate leak, low circulating levels of 1,25 dihydroxy vitamin D3 (calcitriol), elevated alkaline phosphatase, bone hypomineralisation that in adults is presented as a generalised bone softening (osteomalacia) and low serum phosphate. The pathophysiologies of HYP and OHO clearly overlap. In rickets, the defect is a non functional PHEX gene. However, in OHO it is circulating unprocessed phophatonin. The tumours are often difficult to find, and can be extremely difficult or dangerous to resect. Control of phosphate metabolism and bone mineralisation is essential when removal of tumour is contra-indicated. Administration of PHEX to patients to cleave hormone is predicted to be dangerous as other circulating hormones and proteins may also be affected by promiscuous cleavage. Phosphatonin-fragments could instead be designed that have high receptor affinity and bioactivity, such that they would compete effectively with unprocessed tumour-derived circulating hormone.

Other rickets or hypophosphataemic conditions:

There are many causes of rickets besides HYP and OHO, the most common involve abnormalities of vitamin D, but there are causes

such as hypophosphataemia, renal tubular acidosis, use of certain medications, sprue, cystic fibrosis etc. Use of fragments of phosphatonin, and phosphatonin itself may be of use in treating these diseases. Some of the diseases are briefly discussed below (diseases resulting in hyperphosphataemia are potentially treatable by use of the whole hormone).

- Renal transplants and renal osteodystrophy:

  A chronic feature of renal transplantation is the development of a renal phosphate leak (hypophosphataemia), and abnormal bone mineralisation. Phosphatonin fragments would be effective in treating this without the side-effects associated with current medications.

  Osteodystophy (a combination of bone disorders), is usually caused by chronic kidney failure (renal disease). Renal failure will result in death, unless dialysis is given (end stage renal disease). Therefore, patients with osteodystrophy are usually on dialysis therapy. This bone disease, which is also referred to as "renal osteodystrophy," is common in patients on chronic hemodialysis. Secondary hyperparathyroidism develops in most patients with chronic renal failure, and is associated with the histologic finding of osteitis fibrosa cystica. The disease is characterized by growth failure and severe bone deformities in children, especially the very young. The pathogenesis of renal osteodystrophy is related to phosphate retention (hyperphosphataemia), and its effect on calcium and calcitriol metabolism, in addition to roles played by metabolic acidosis,

cytokines, and degradation of parathyroid hormone. Treatment includes restriction of dietary phosphorous intake, phosphate binders, and use of active metabolites of vitamin D. In this context addition of unprocessed hormone would be a powerful means of controlling phosphate levels, and would lead to bone healing. If receptors for phosphatonin are expressed in a range of tissues as well as the kidney, then the potential for treating patients with end stage renal disease exists (complete loss of kidney function).

- Osteoporosis/bone mineral loss:

  Post-menopausal women are prone to loss of bone mineral with consequent damage to the integrity of the skeleton. The cause is unknown but is likely to involve a complex interaction of genetic and environmental factors. Current research is focussed on refining statistical models to analyse multifactorial diseases such as osteoporosis.

  The use of phosphatonin-derivative fragment(s) would help in the treatment of this disease by potentially reversing the bone mineral loss. Moreover, the bioactive peptides could be modified to increase potency and specificity of action.

- Pagets disease of bone:

  Pagets disease occurs due to asynchronous bone re-modelling. Bone mineralisation (mediated by osteoblasts), and bone resorption (mediated by osteoclasts), are out of step. Excessive osteoclast resorptive activity occurs (predominantly in the early

resorptive phase), and bone marrow is replaced by fibrous tissue and disorganized trabeculae. Although the cause is unknown, administration of peptide derivatives of phosphatonin may help in the treatment of the disease.

Pharmaceutical Compositions

Pharmaceutical compositions may be formulated comprising a polypeptide according to the present invention optionally incorporating a pharmaceutically-acceptable exipient, diluent or carrier. The exact nature and quantities of the components of such compositions may be determined empirically and will depend in part upon the route of administration of the composition. Routes of administration to patients include oral, buccal, sublingual, topical (including ophthalmic), rectal, vaginal, nasal and parenteral (including intravenous, intraarterial, intramuscular, subcutaneous and intraarticular). In order to avoid unwanted proteolysis, a parenteral route is preferred.

Suitable dosages of a molecule of the present invention will vary, depending upon factors such as the disease or disorder to be treated, the route of administration and the age and weight of the individual to be treated. For instance for parenteral administration, a daily dosage of from 0.1µg to 1.5 mg/kg of a molecule of the invention may be suitable for treating a typical adult. More suitably the dose might be 1µg to 150 µg. Accordingly, it is envisaged that the active polypeptide ingredient may be given in a dose range of from 0.01 to 100 mg, typically 0.1 to 10 mg, on a daily basis for an adult human.

Compositions for parenteral administration for example will usually comprise a solution of the molecule dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used such as water, buffered water, 0.4% saline, 0.3% glycine etc. Such solutions should advantageously be sterile and generally free of aggregate and other particulate matter. The compositions may contain pharmaceutically acceptable buffers to adjust pH, or alter toxicity, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of molecule in these formulations can vary widely, for example from less than about 0.5% to as much as 15 or 20% by weight and could be selected as appropriate by a skilled person.

Typical pharmaceutical compositions are described in detail in Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980). For example pharmaceutical compositions for injection could be made up to contain 1 ml sterile buffered water, and 50 mg of molecule. A typical composition for infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of molecule. Actual methods for preparing compositions will be known or apparent to those skilled in the art. Approaches to formulation and administration of polypeptide pharmaceutical compositions are well-known to those skilled in this art and are discussed, for example, by P. Goddard in Advanced Drug Delivery Reviews, 6(1991) 103-131.

Table 1

	Site (on Figure 8)	Motif
Protein Kinase C phosphorylation	8-10	SNK
	77-79	TPR
	118-120	THR
	203-205	TKK
	228-230	TAK
	311-313	STR
	312-314	TRK
	319-321	SNR
	384-386	STR
	403-405	SNR
	408-410	SSR
	409-411	SRR
Casein Kinase II phosphorylation	8-11	SNKE
	139-142	SDFE
	177-180	TGPD
	194-197	SEAE
	199-202	THLD
	224-227	TRDE
	228-231	TAKE
	238-241	SLVE
	325-328	TLNE
	423-426	SSSE
	425-428	SESD
	427-430	SDGD
CAMP- & cGMP-dependent protein kinase phosphorylation	405-408	RRFS
Tyrosine Kinase phosphorylation	40-47	KLHDQEEY

Table 1 (continued)

	Site (on Figure 8)	**Motif**
Myristoylation	16-21	GLRMSI
	143-148	GSGYTD
	119-224	GNTIGT
	266-271	GSQNAH
	291-296	GSSDAA
	315-320	GVDHSN
	389-394	GMPQGK
Amidation	370-373	HGRK

CLAIMS:

1. An isolated polypeptide having phosphatonin activity.

2. A polypeptide according to claim 1, which has an approximate molecular weight of 53 to 60 kDa as measured on SDS-PAGE.

3. A polypeptide according to claim 1 or claim 2, which has an approximate molecular weight of 200kDa as measured on bis- tris SDS-PAGE at pH7.

4. A polypeptide according to any one of claims 1 to 3, which is glycosylated.

5. A polypeptide according to any one of the preceding claims, which is obtainable following purification from Saos-2 cells (Deposit No. ECACC 89050205).

6. A polypeptide according to any one of the preceding claims, which comprises the amino acid sequence of Figure 8, optionally including mutations or deletions which do not substantially affect the activity thereof.

7. A polypeptide according to any one of the preceding claims, which comprises phosphatonin in substantially pure form.

8. A polypeptide comprising a bioactive fragment of a polypeptide according to any one of the preceding claims.

9. A polypeptide according to claim 8, which is capable of regulating phosphate metabolism.

10. A polypeptide according to claim 8 or claim 9, which has a cell attachment motif.

11. A polypeptide according to claim 10, wherein the cell attachment motif comprises RGD.

12. A polypeptide according to any one of claims 8 to 11, which has a glycosaminoglycan attachment motif.

13. A polypeptide according to claim 12, wherein the glycosaminoglycan attachment motif comprises SGDG.

14. A polypeptide according to any one of the preceding claims, which is capable of down-regulating renal sodium dependent phosphate co-transport.

15. A polypeptide according to any one of the preceding claims, which is further capable of up-regulating renal 25-hydroxy vitamin D3-24-hydroxylase.

16. A polypeptide according to any one of the preceding claims, which is further capable of down-regulating renal 25-hydroxy-D-1-α -hydroxylase.

17. A polypeptide according to any one of the preceding claims, which further comprises at least one intact PHEX cleavage site.

18. A polypeptide according to claim 17, wherein the PHEX cleavage site comprises ADAVDVS.

19. A polypeptide according to any one of claims 8 to 13, which is capable of up-regulating renal sodium dependent phosphate co-transport.

20. A polypeptide according to any one of claims 8 to 13 or 19, which is further capable of down-regulating renal 25-hydroxy vitamin D3-24-hydroxylase.

21. A polypeptide according to any one of claims 8 to 13, 19 or 20, which is further capable of up-regulating renal 25-hydroxy-D-1 -hydroxylase.

22. A polypeptide according to any one of claims 8 to 13, or 19 to 21, which comprises no more than approximately the first 235 residues of the amino acid sequence of Figure 8.

23. A polypeptide according to any one of claims 8 to 13, or 19 to 22 which is obtainable by proteolytic cleavage of phosphatonin by a PHEX metallopeptidase.

24. An isolated polynucleotide encoding a polypeptide according to any one of the preceding claims.

25. A polynucleotide according to claim 24, which comprises DNA.

26. A polynucleotide according to claim 24 or claim 25, which comprises the nucleotide sequence of Figure 8, optionally including one or more mutations or deletions which do not substantially affect the activity of the polypeptide encoded thereby.

27. A recombinant polynucleotide comprising a vector incorporating a polynucleotide according to any one of claims 24 to 26.

28. A recombinant polynucleotide according to claim 27, wherein the vector comprises an expression vector.

29. A cell capable of expressing a polypeptide according to any one of claims 1 to 23, which comprises a recombinant host cell incorporating a polynucleotide according to any one of claims 24 to 26.

30. A cell according to claim 29, wherein the recombinant host cell includes a recombinant polynucleotide according to claim 27 or claim 28.

31. A polypeptide according to any one of claims 1 to 13, which comprises a fusion polypeptide encoded by a recombinant polynucleotide according to claim 27 or claim 28, a part of which is encoded by the vector.

32. A polypeptide according to any one of claims 1 to 23, or 31, for use as a medicament.

33. Phosphatonin and PHEX metallopeptidase as a combined preparation for simultaneous, separate or sequential use as a medicament.

34. Use of a polypeptide according to any one of claims 1 to 23 or 31, for the preparation of a medicament for treatment of a disorder of phosphate metabolism.

35. Use of a polypeptide according to any one of claims 1 to 18, for the preparation of a medicament for the treatment of hyperphosphataemia.

36. Use of a polypeptide according to any one of claims 1 to 18, for the preparation of a medicament for the treatment of renal osteodystrophy, secondary hyperparathyrodism or osteitis fibrosa cystica.

37. Use of a polypeptide according to any one of claims 1 to 13, 19 to 23 or 31, for the preparation of a medicament for the treatment of hypophosphataemia.

38. Use of a polypeptide according to any one of claims 1 to 13 or 19 to 23, for the preparation of a medicament for the treatment of X-linked rickets, hereditary hypophosphataemic

rickets with hypercalcuria (HHRH), hypomineralised bone lesions, stunted growth in juveniles, oncogenic hypophosphataemic osteomalacia, renal phosphate leakage, renal osteodystrophy, osteoporosis, vitamin D resistant rickets, end organ resistance, renal Fanconi syndrome, autosomal rickets, Paget's disease, kidney failure or hypophosphataemia in a renal dialysis/pre-dialysis patient, renal tubular acidosis, cystic fibrosis and sprue.

39. Use of a polypeptide according to any one of claims 8 to 13, 19 to 23 or 31, for the manufacture of a medicament for the treatment of a bone mineral loss disorder.

40. Use of phosphatonin and PHEX metallopeptidase for the manufacture of a combined preparation for simultaneous, separate or sequential use for the treatment of a disorder of phosphate metabolism.

41. Use of a transformed osteoblast or bone cell line capable of phosphatonin overexpression for the production of phosphatonin.

42. Use according to claim 41 wherein the cell line is an Saos-2 cell (Deposit No. ECACC 89050205) or a U-2 OS cell (Deposit No. ATCC HTB-96).

43. A pharmaceutical composition comprising a polypeptide according to any one of claims 1 to 23 or 31, and a pharmaceutically-acceptable exipient, diluent or carrier.

09-Ju-81 M

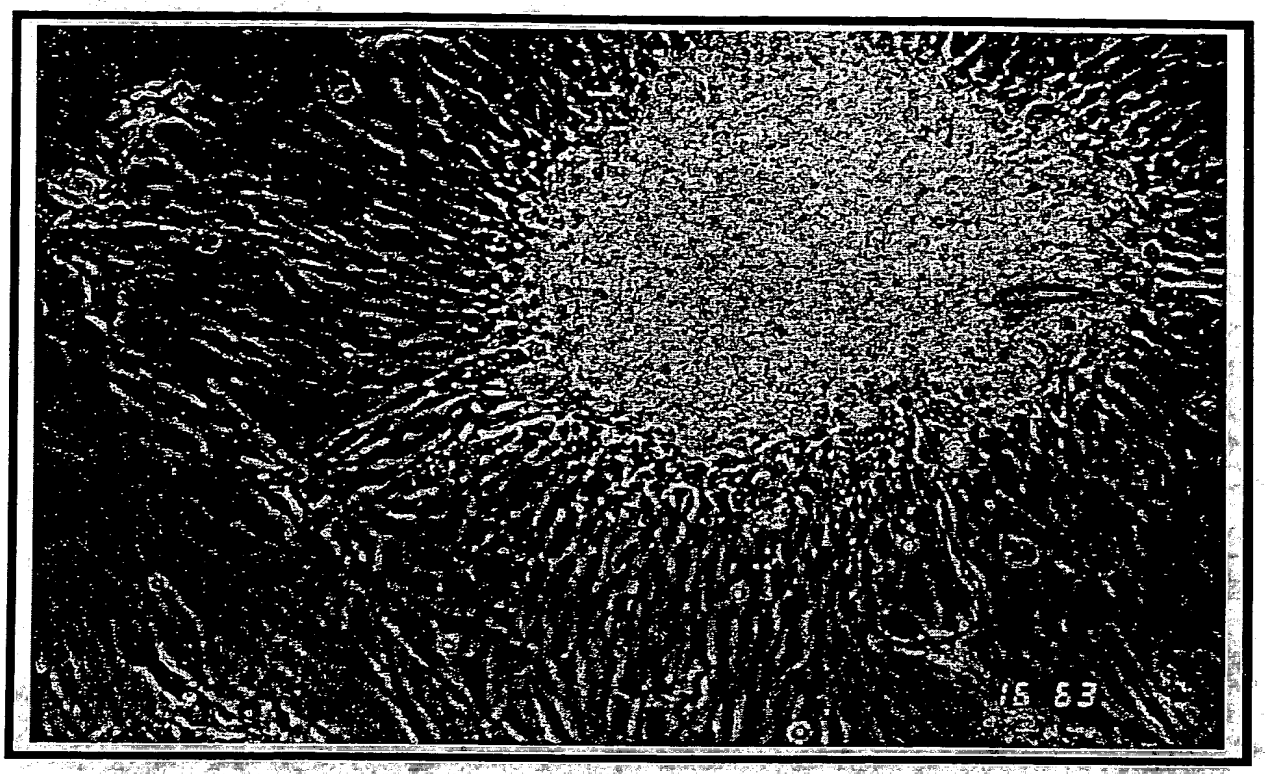


FIGURE 1

09-09-91 M

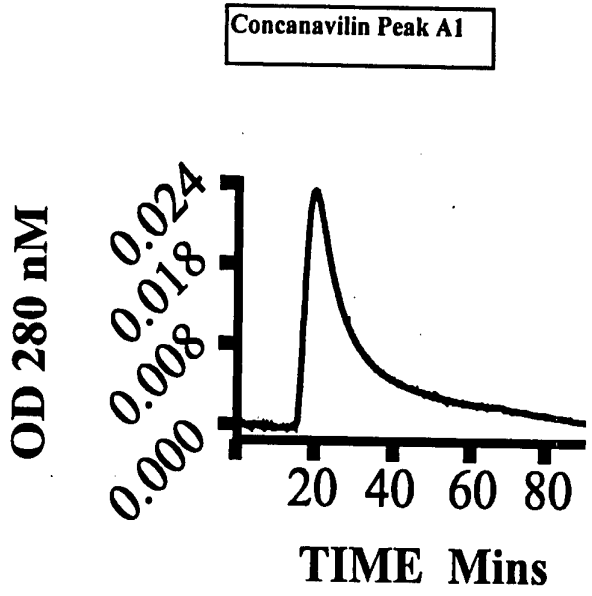


Figure 2a

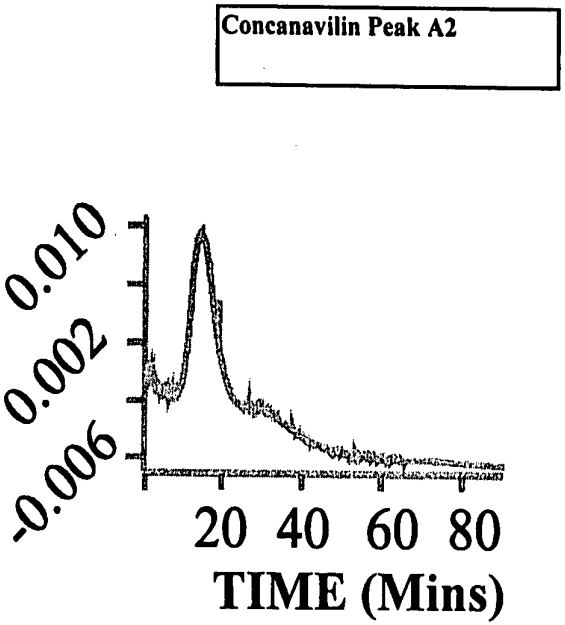
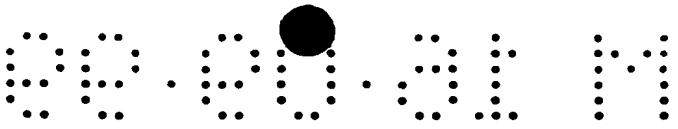


Figure 2b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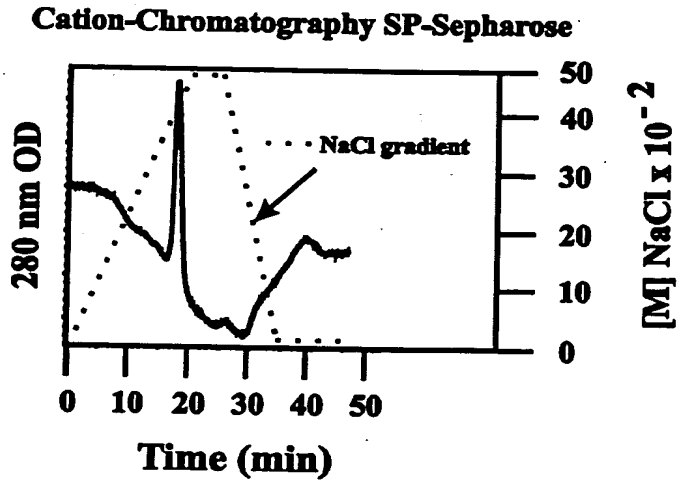

Cation-Chromatography SP-Sepharose
280 nm OD
NaCl gradient
[M] NaCl x 10-2
50
40
30
20
10
0
0 10 20 30 40 50
Time (min)

FIGURE 3

09-Aug-21 M

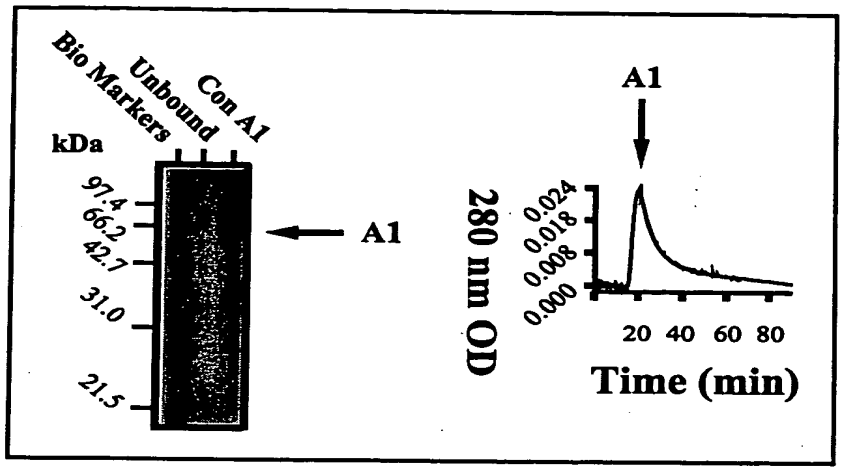
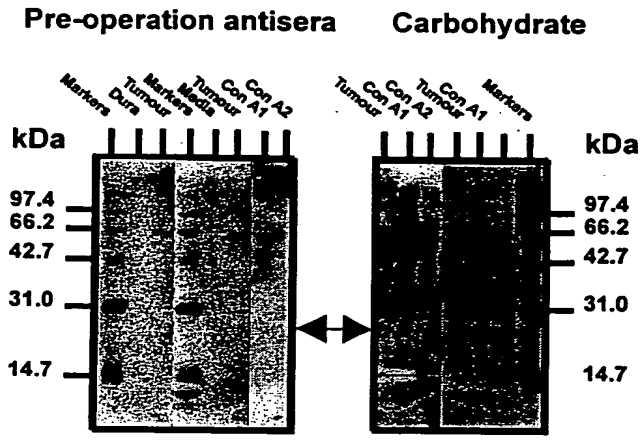

Bio Markers
Unbound
Con A1
kDa
97.4
66.2
42.7
31.0
21.5
A1
A1
280 nm OD
0.024
0.016
0.008
0.000
20 40 60 80
Time (min)

FIGURE 4

09-09-81 M


Pre-operation antisera
Carbohydrate
Markers
Dura
Tumour
Markers
Media
Tumour
Con A1
Con A2
Tumour
Con A1
Con A2
Tumour
Con A1
Markers
kDa
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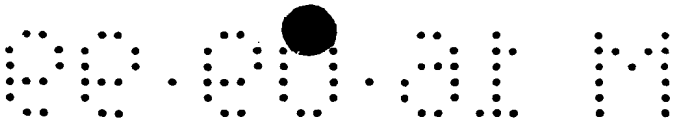
97.4
66.2
42.7
31.0
14.7

FIGURE 5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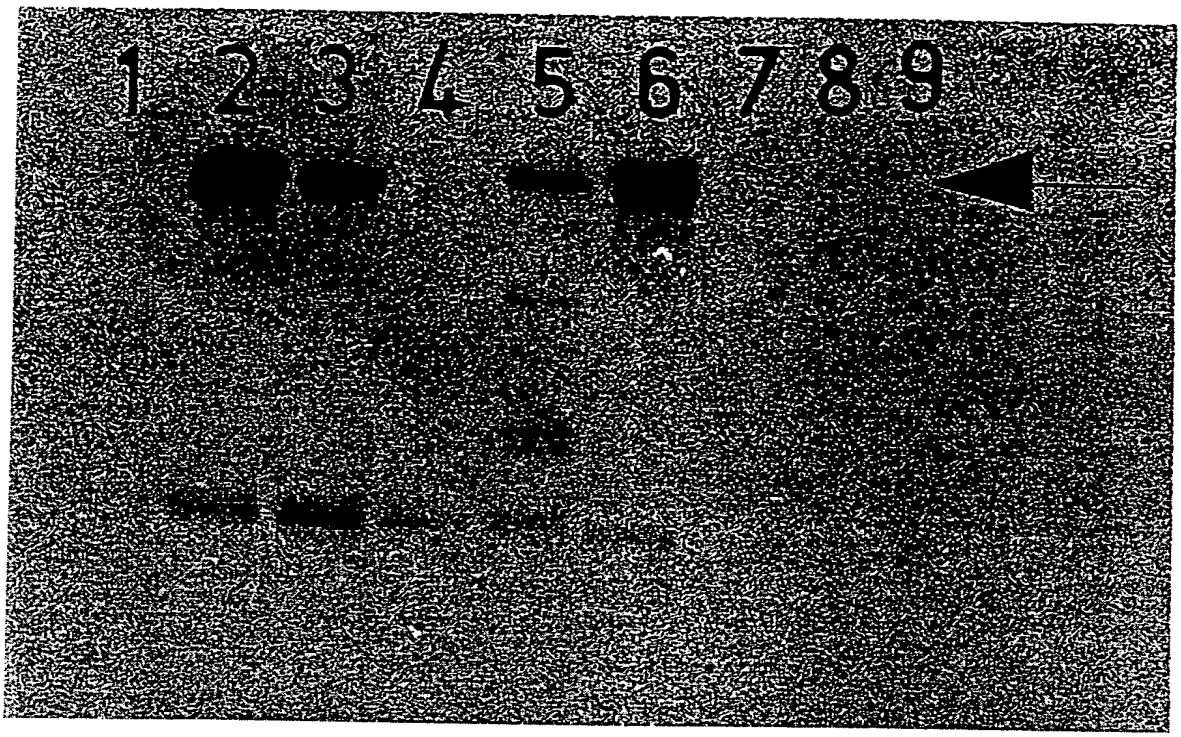
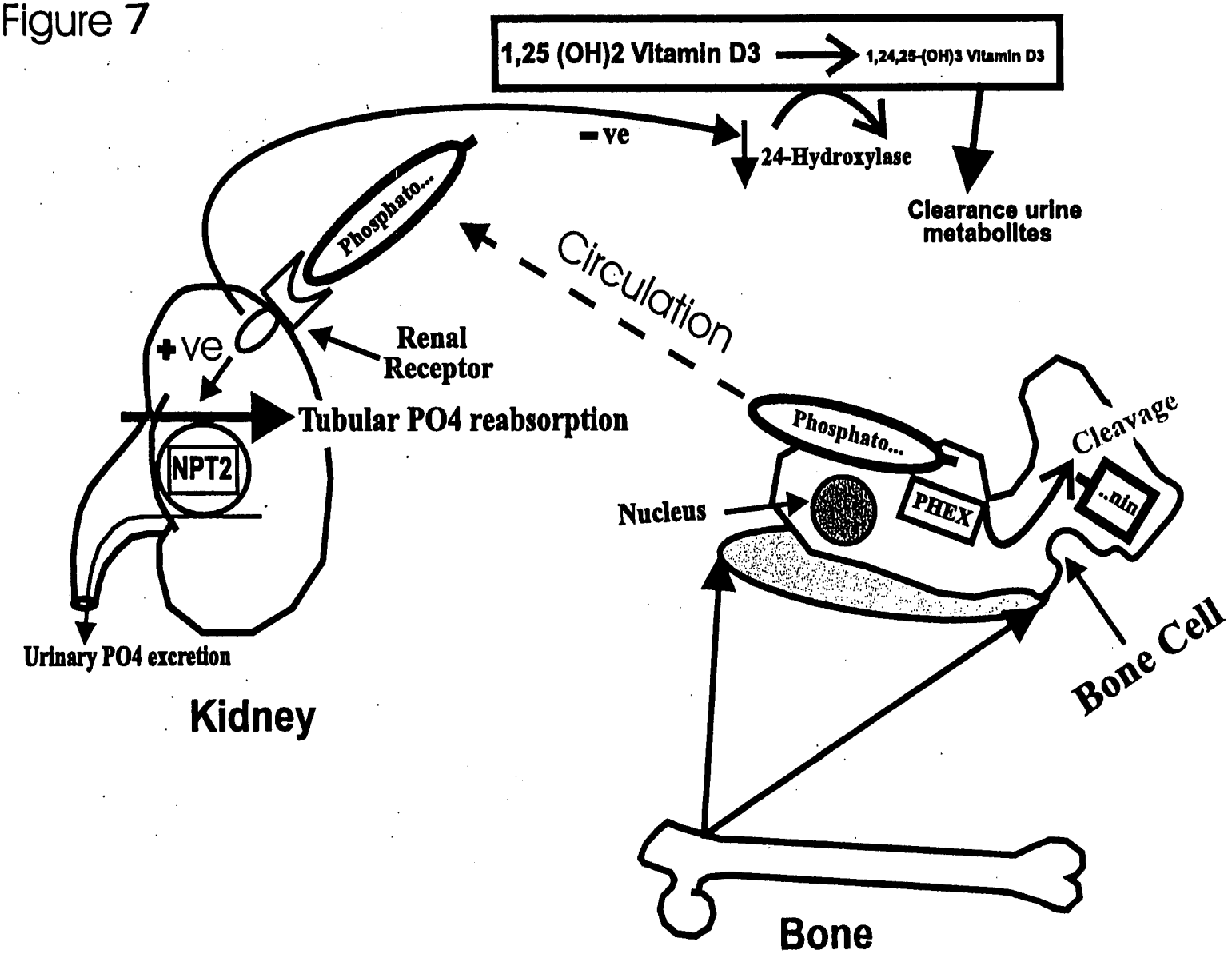


FIGURE 6

09-Sep-81 M

Figure 7


1,25 (OH)2 Vitamin D3
1,24,25-(OH)3 Vitamin D3
-ve
24-Hydroxylase
Clearance urine metabolites
Phosphato...
Circulation
+ve
Renal Receptor
Tubular PO4 reabsorption
NPT2
Urinary PO4 excretion
Kidney
Phosphato...
Cleavage
Nucleus
PHEX
..nin
Bone Cell
Bone

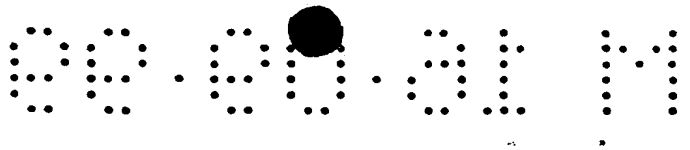

09:09:31 M

FIGURE 8

```
 V  N  K  E  Y  S  I  S  N  K  E  N  T  H  N  G  L  R  M  S
GTGAATAAAGAATATAGTATCAGTAACAAAGAGAATACTCACAATGGCCTGAGGATGTCA      60

 I  Y  P  K  S  T  G  N  K  G  F  E  D  G  D  D  A  I  S  K
ATTTATCCTAAGTCAACTGGGAATAAAGGGTTTGAGGATGGAGATGATGCTATCAGCAAA     120

 L  H  D  Q  E  E  Y  G  A  A  L  I  R  N  N  M  Q  H  I  M
CTACATGACCAAGAAGAATATGGCGCAGCTCTCATCAGAAATAACATGCAACATATAATG     180

 G  P  V  T  A  I  K  L  L  G  E  E  N  K  E  N  T  P  R  N
GGGCCAGTGACTGCGATTAAACTCCTGGGGGAAGAAAACAAAGAGAACACACCTAGGAAT     240

 V  L  N  I  I  P  A  S  M  N  Y  A  K  A  H  S  K  D  K  K
GTTCTAAACATAATCCCAGCAAGTATGAATTATGCTAAAGCACACTCGAAGGATAAAAAG     300

 K  P  Q  R  D  S  Q  A  Q  K  S  P  V  K  S  K  S  T  H  R
AAGCCTCAAAGAGATTCCCAAGCCCAGAAAAGTCCAGTAAAAAGCAAAAGCACCCATCGT     360

 I  Q  H  N  I  D  Y  L  K  H  L  S  K  V  K  K  I  P  S  D
ATTCAACACAACATTGACTACCTAAAACATCTCTCAAAAGTCAAAAAAATCCCCAGTGAT     420

 F  E  G  S  G  Y  T  D  L  Q  E  R  G  D  N  D  I  S  P  F
TTTGAAGGCAGCGGTTATACAGATCTTCAAGAGAGAGGGGACAATGATATATCTCCTTTC     480

 S  G  D  G  Q  P  F  K  D  I  P  G  K  G  E  A  T  G  P  D
AGTGGGGACGGCCAACCTTTTAAGGACATTCCTGGTAAAGGAGAAGCTACTGGTCCTGAC     540

 L  E  G  K  D  I  Q  T  G  F  A  G  P  S  E  A  E  S  T  H
CTAGAAGGCAAAGATATTCAAACAGGGTTTGCAGGCCCAAGTGAAGCTGAGAGTACTCAT     600

 L  D  T  K  K  P  G  Y  N  E  I  P  E  R  E  E  N  G  G  N
CTTGACACAAAAAAGCCAGGTTATAATGAGATCCCAGAGAGAGAAGAAAATGGTGGAAAT     660

 T  I  G  T  R  D  E  T  A  K  E  A  D  A  V  D  V  S  L  V
ACCATTGGAACTAGGGATGAAACTGCGAAAGAGGCAGATGCTGTTGATGTCAGCCTTGTA     720

 E  G  S  N  D  I  M  G  S  T  N  F  K  E  L  P  G  R  E  G
GAGGGCAGCAACGATATCATGGGTAGTACCAATTTTAAGGAGCTCCCTGGAAGAGAAGGA     780

 N  R  V  D  A  G  S  Q  N  A  H  Q  G  K  V  E  F  H  Y  P
AACAGAGTGGATGCTGGCAGCCAAAATGCTCACCAAGGGAAGGTTGAGTTTCATTACCCT     840

 P  A  P  S  K  E  K  R  K  E  G  S  S  D  A  A  E  S  T  N
CCTGCACCCTCAAAAGAGAAAAGAAAAGAAGGCAGTAGTGATGCAGCTGAAAGTACCAAC     900

 Y  N  E  I  P  K  N  G  K  G  S  T  R  K  G  V  D  H  S  N
TATAATGAAATTCCTAAAAATGGCAAAGGCAGTACCAGAAAGGGTGTAGATCATTCTAAT     960

 R  N  Q  A  T  L  N  E  K  Q  R  F  P  S  K  G  K  S  Q  G
AGGAACCAAGCAACCTTAAATGAAAAACAAAGGTTTCCTAGTAAGGGCAAAAGTCAGGGC    1020

 L  P  I  P  S  R  G  L  D  N  E  I  K  N  E  M  D  S  F  N
CTGCCCATTCCTTCTCGTGGTCTTGATAATGAAATCAAAAACGAAATGGATTCCTTTAAT    1080

 G  P  S  H  E  N  I  I  T  H  G  R  K  Y  H  Y  V  P  H  R
GGCCCCAGTCATGAGAATATAATAACACATGGCAGAAAATATCATTATGTACCCCACAGA    1140

 Q  N  N  S  T  R  N  K  G  M  P  Q  G  K  G  S  W  G  R  Q
```

Figure 8 (continued)

```
CAAAATAATTCTACACGGAATAAGGGTATGCCACAAGGGAAAGGCTCCTGGGGTAGACAA    1200

 P  H  S  N  R  R  F  S  S  R  R  R  D  D  S  S  E  S  S  D
CCCCATTCCAACAGGAGGTTTAGTTCCCGTAGAAGGGATGACAGTAGTGAGTCATCTGAC    1260

 S  G  S  S  S  E  S  D  G  D  *
AGTGGCAGTTCAAGTGAGAGCGATGGTGACTAGTCCACCAGGAGTTCCCAGCGGGGTGAC    1320

AGTCTGAAGACCTCGTCACCTGTGAGTTGATGTAGAGGAGAGCCACCTGACAGCTGACCA    1380

GGTGAAGAGAGGATAGAGTGAAGAACTGAGTGAGCCAAGAATCCTGGTCTCCTTGGGGGA    1440

ATTTTTGCTATCTTAATAGTCACAGTATAAAATTCTATTAAAGGCTATAATGTTTTTAAG    1500

CAAAAAAAAATCATTACAGATCTATGAAATAGGTAACATTTGAGTAGGTGTCATTTAAAA    1560

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CAATATCTCTCGATGATAAAAAAAAAAAAAAAAA    1655
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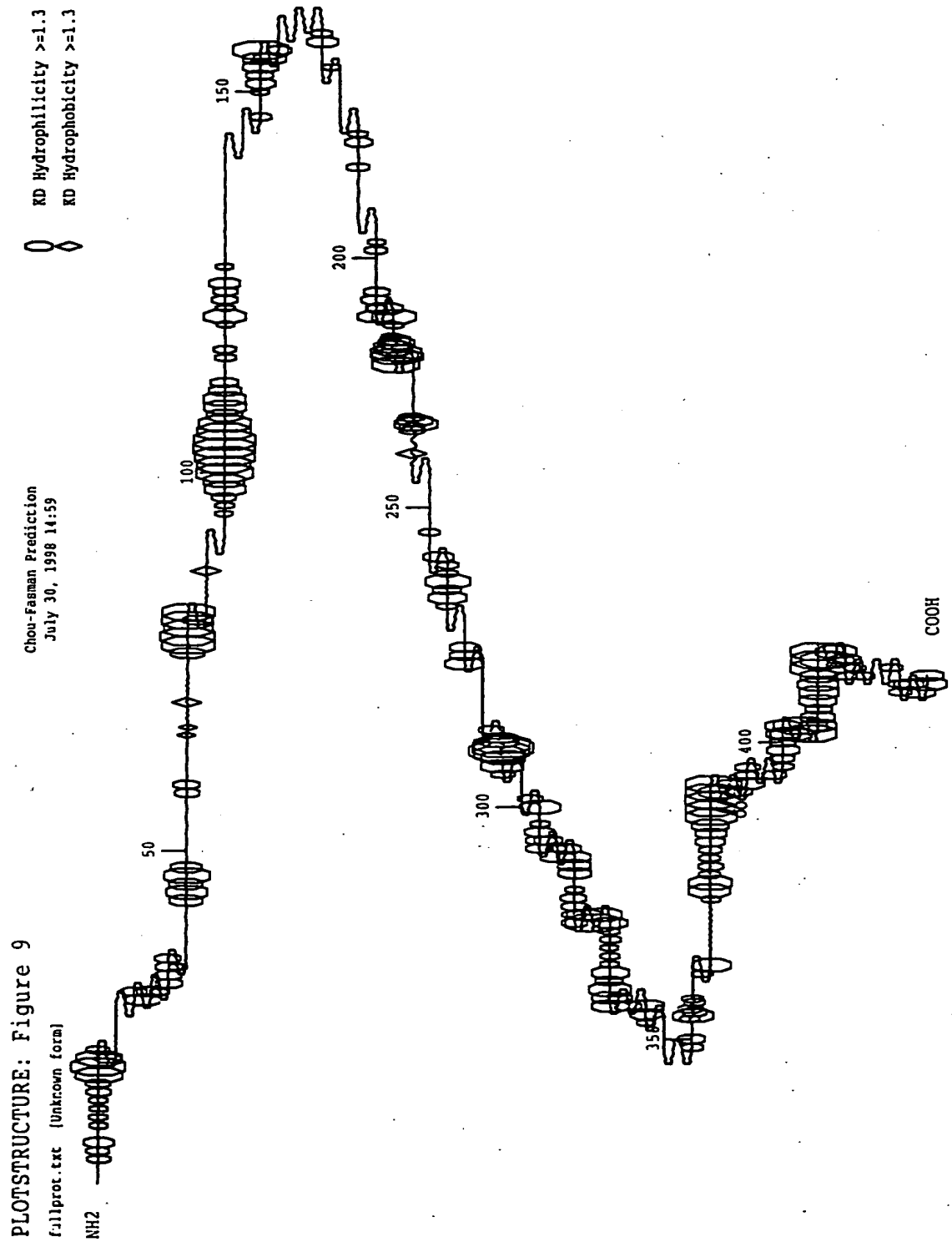
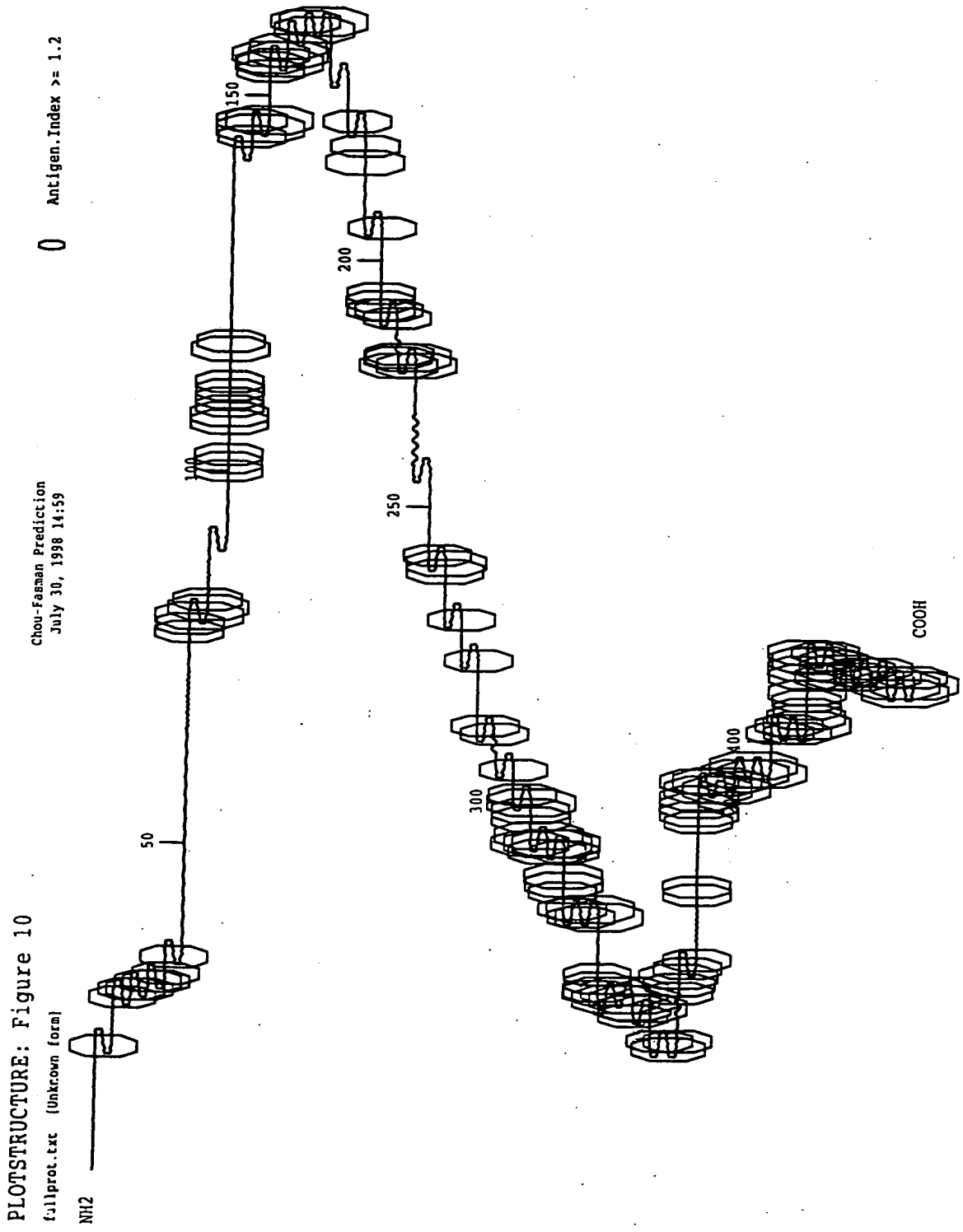
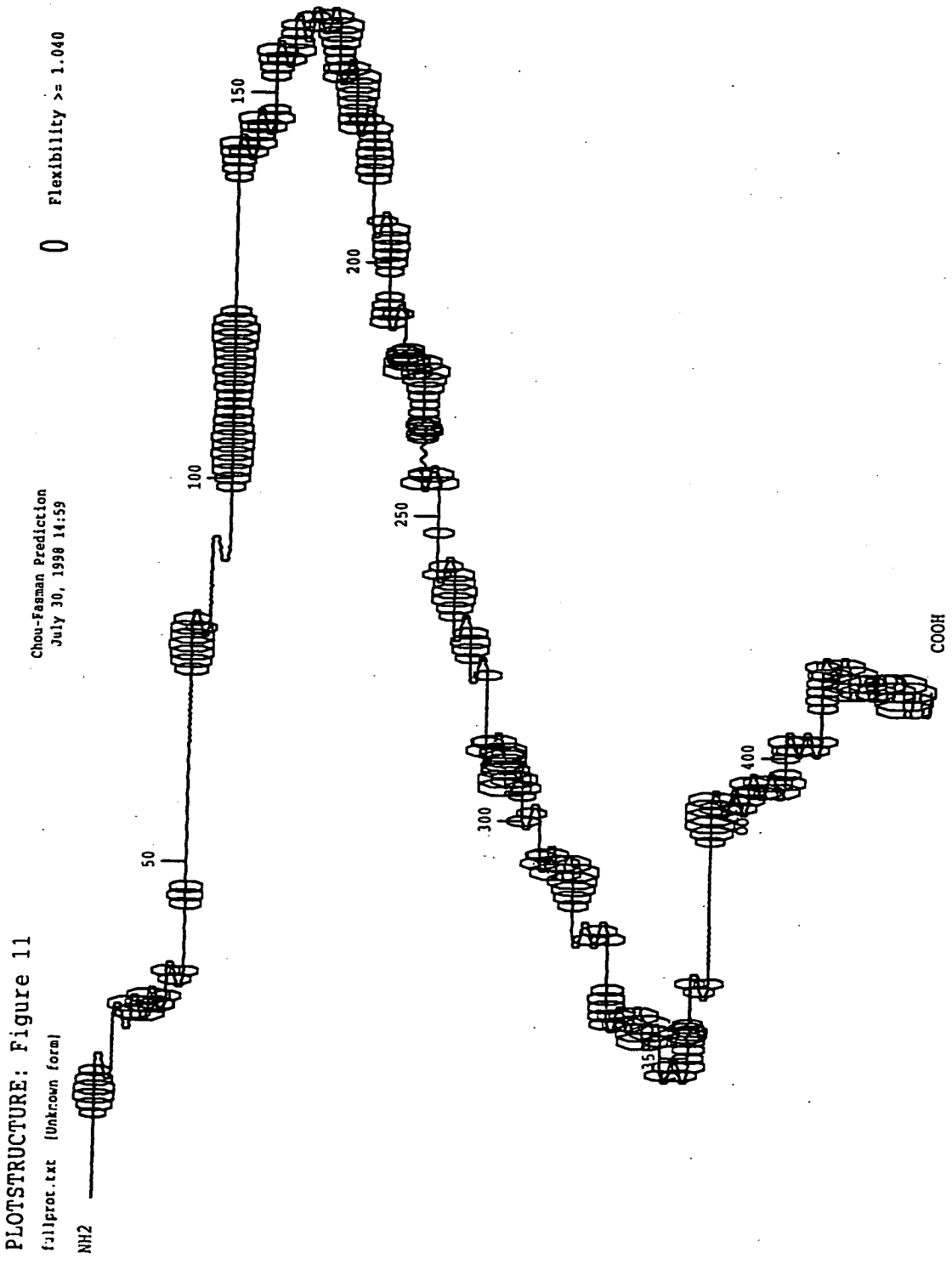
09-04-21 M


PLOTSTRUCTURE: Figure 9
fullprot.txt (Unknown form)
NH2
COOH
Chou-Fasman Prediction
July 30, 1998 14:59
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
50
100
150
200
250
300
350
400

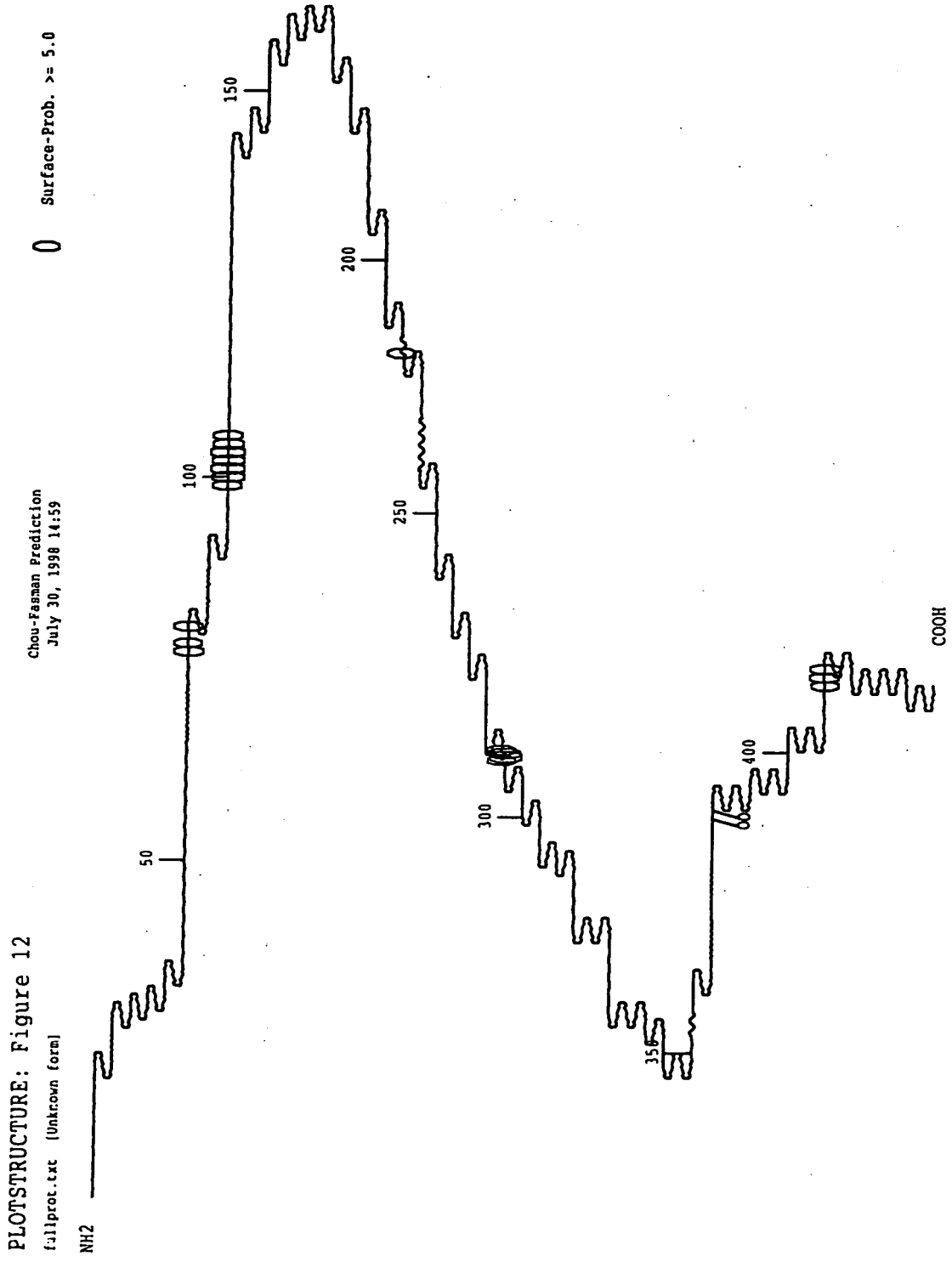
08-Aug-81 M


PLOTSTRUCTURE: Figure 10
fullprot.txt (Unknown form)
Chou-Fasman Prediction
July 30, 1998 14:59
Antigen.Index >= 1.2
0
NH2
50
100
150
200
250
300
400
COOH

09-Sv-81 M


PLOTSTRUCTURE: Figure 11
fullprot.txt (Unknown form)
Chou-Fasman Prediction
July 30, 1998 14:59
Flexibility >= 1.040
NH2
COOH
50
100
150
200
250
300
350
400

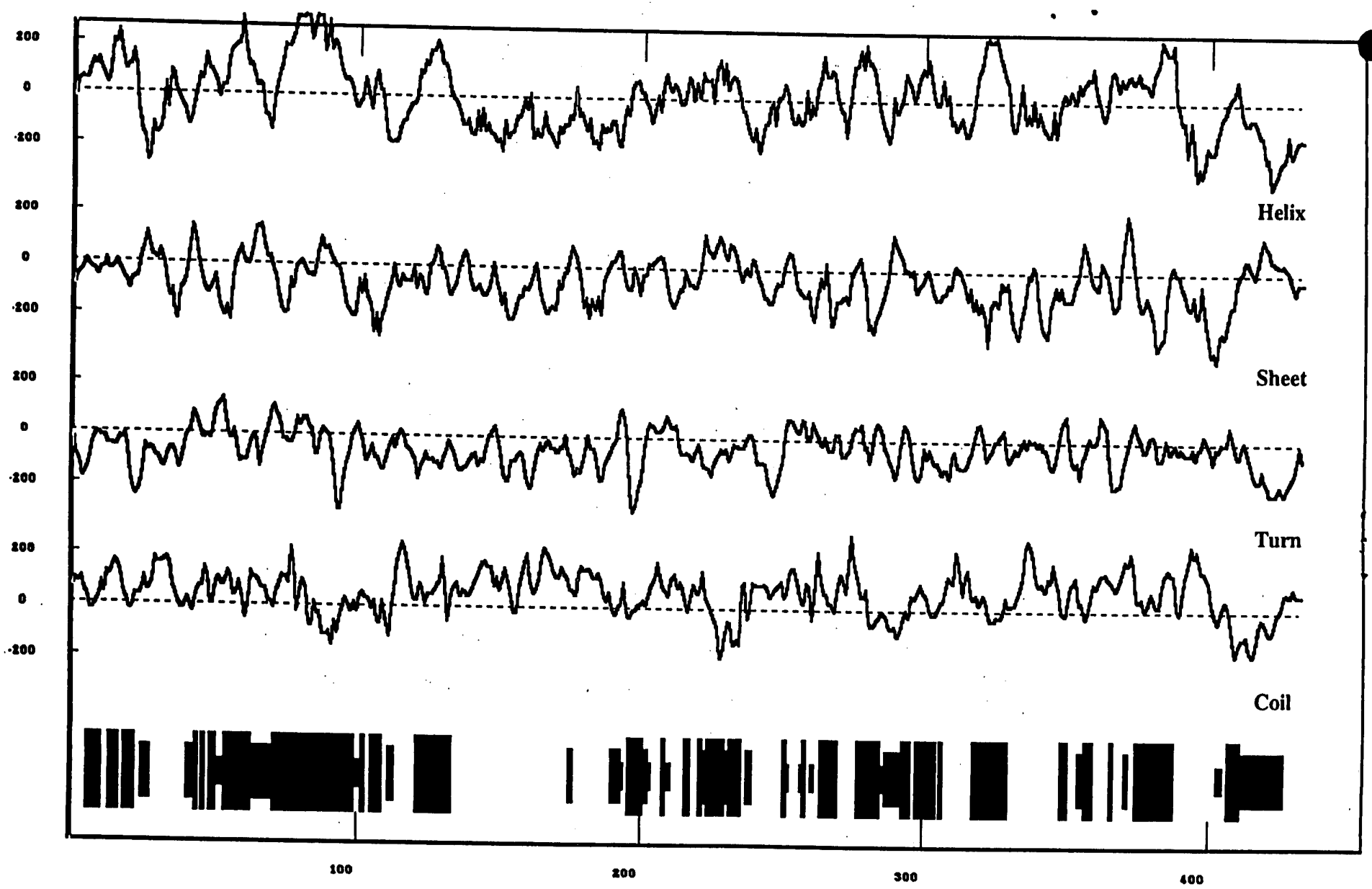
09-09-81 M


PLOTSTRUCTURE: Figure 12
fullprot.txt (Unknown form)
Chou-Fasman Prediction
July 30, 1998 14:59
0 Surface-Prob. >= 5.0
NH2
50
100
150
200
250
300
350
400
COOH

09:09:31 M

1 AA VNKEYS State CCCCCH Helix -30 Sheet -99 Turn -10 Coil 65

Figure 13:



14/14

09-09-81 M